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Intramolecular Catalysis of Sulfate Ester Hydrolysis. A Model for Aryl Sulfate Sulfohydrolase*

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ABSTRACT: The hydrolysis of 2-(4(5)-imidazolyl)phenyl sulfate (I) was studied as a possible model for aryl sulfate sulfohydrolase. The pH–rate profile may be divided into two regions: (1) at pH 1–3 the compound undergoes conventional A-1 acid-catalyzed hydrolysis, (2) at pH 4–7 the neighboring imidazole moiety catalyzes the reaction. The compound which would result from intramolecular nucleophilic attack by imidazole, 4(5)-(2′-hydroxyphenyl)imidazole *N*-sulfate, was synthesized and its solvolytic behavior compared with the initial substrate. Although trapping experiments designed to detect the intermediacy of the *N*-sulfate do not entirely exclude that mode of catalysis, the hydrolysis of I at pH 4–7 appears to involve mainly intramolecular general acid catalysis. Comparison of I with salicyl sulfate and other compounds capable of hydrogen bonding indicates rate accelerations can be rationalized in terms of transition state stability as manifested by ΔpK_a for the corresponding phenolic products.

The arylsulfatase enzymes which catalyze the hydrolysis of aryl sulfates have been found in several microorganisms and in higher plants and mammals (Roy, 1960, and references therein). Although the arylsulfatases A and B of mammalian liver have been extensively studied (Roy, 1960, and references therein), the physiological functions of these aryl sulfatases remain unknown. Recent *in vitro* searches for natural substrates have led to the postulation that cerebroside 3-sulfate may be degraded *in vivo* by lysosomal arylsulfatases (Mehl and Jatzkewitz, 1968), but as yet there is only indirect evidence (Choy and Cravioto, 1968). The function of a related but distinct class of enzymes, the steroidal sulfatases, is more visible since their importance in the *in vivo* synthesis of estrogens *via* the simple hydrolysis of estrogen sulfates during pregnancy (Benagiano *et al.*, 1967) and in the normal state (Sandberg and Jenkins, 1966) has been demonstrated.

The nature of the catalytic groups at the active site of these enzymes is unknown. Detailed studies with the sulfatase of *A. metalcaligenes* (Dodgson, *et al.*, 1956) have revealed that both the rate of hydrolysis and binding of substrate to the enzyme are increased by the introduction of electron withdrawing substituents in the aryl sulfates, and that the O–S bond of the aryl sulfate is cleaved (Spencer, 1958). A recent study of the action of group-specific protein reagents on sulfatase A of ox liver (Jerfy and Roy, 1969) has shown that neither SH groups nor amino groups are involved in the hydrolysis reaction but that tyrosyl residues are essential for activity of the enzyme, which is inactivated by treatment with *N*-acetylimidazole or with tetranitromethane. Histidyl residues are implicated in the mechanism of action by spontaneously reversible inactivation of the enzyme with acetic anhydride and inactivation by photooxidation in the presence of Rose Bengal.

Some insight into the reaction catalyzed by sulfatase A might be gained by studying the hydrolysis of a molecular model which incorporates the essential features of tyrosyl and histidyl residues. For this reason, we have studied the hydrolysis of 2-(4(5)imidazolyl)phenyl sulfate.

Experimental Section

4-(2′-Hydroxyphenyl)imidazole was prepared by the method of Pandit and Bruice (1960), mp 179–180° uncorrected. *Anal.* Calcd for $C_9H_8N_2O$: C, 67.55; H, 5.04; N, 17.51. Found: C, 67.30; H, 5.07; N, 17.44. Selective sulfation of the phenolic oxygen or the imidazole nitrogen depends upon the conditions employed.

2-(4(5)-Imidazolyl)phenyl Sulfate. 4-(2′-Hydroxyphenyl)-imidazole (1.0 g, 6.2 mmoles) and fresh pyridine–sulfur trioxide complex (1.0 g, 6.2 mmoles) were dissolved in 20 ml of anhydrous dimethylformamide in a 35-ml round-bottom flask fitted with a drying tube, and warmed at 80°. After 3.5 hr, the solution was cooled in an ice bath and poured into 250 ml of ice water, and 5% KOH added dropwise to bring the solution to pH 8. Saturated barium hydroxide solution was added dropwise until all inorganic sulfate precipitated, which was filtered off and the filtrate washed with two 200-ml portions of ether. The aqueous phase was concentrated to 50 ml under vacuum, passed through an NH_4^+ form of a Rexyn-101 (H^+) column, and concentrated under vacuum to yield an orange liquid.

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Addition of 10–25 ml of anhydrous methanol followed by 500–800 ml of anhydrous ether gave the ammonium salt of the compound as an off-white precipitate; yield: 0.72 g, 45.3%. The product was reprecipitated twice by dissolving it in anhydrous methanol and precipitating with anhydrous ether. This material was dried at room temperature for 1.5 hr under vacuum, at which time ammonia is presumably lost, since the dried material, mp 214–215°, gives an analysis consistent with the formation of the free sulfate, possibly in its zwitterionic form. *Anal.* Calcd for $C_9H_8N_2O_4S$: C, 44.99; H, 3.35; N, 11.66. Found: C, 44.77; H, 3.56; N, 11.75. Descending paper chromatography (Whatman paper No. 4; solvent, 1-propanol–ammonium hydroxide–water 6:3:1; short-wave ultraviolet light) showed the material to be homogeneous, and the ultraviolet spectrum at pH 12 showed a phenolate anion absorption of less than 0.7% (ϵ_{max} 13,520 at 260 mμ; ϵ 82 at 320 mμ). The infrared spectrum (Nujol) showed peaks at 1230–1280 (broad multiplet), 1210 (sharp), 1169 (sharp), 1117 (sharp), 1095 (sharp), 1062 (sharp), 1045 (strong), 1037 (shoulder), and 775 cm^{-1} (strong). Complete hydrolysis at pH 2–3 yielded inorganic sulfate quantitatively (±3%).

4(5)-(2′-Hydroxyphenyl)imidazole N-sulfate was prepared by the general method of Weidenhagen *et al.* (1937) for *N*-sulfates. 4-(2′-Hydroxyphenyl)imidazole (1.0 g, 6.2 mmoles) was added to 50 ml of H_2O to which 0.5 g of K_2CO_3 and 0.5 g of $KHCO_3$ had been added to bring the pH to 8–9, and the mixture was cooled to 0°. Fresh pyridine–sulfur trioxide complex (4.0 g, 24.9 mmoles) was added and the mixture stirred at 0°, adding solid K_2CO_3 and $KHCO_3$ periodically to maintain the pH at 8–9. After 5 hr, the remaining solid material was filtered off and later recrystallized from water to give 0.7 g of unreacted starting material. The filtrate was stripped to dryness under vacuum and 50 ml of anhydrous ethanol added. After filtering off the inorganic salts, anhydrous ether was added to force precipitation; 0.25 g (14.5%) of an off-white material was collected. Purification consisted of dissolving the material in cold anhydrous methanol and adding a small amount of anhydrous ether to cause precipitation of a yellow hydroscopic impurity, which was filtered off. Additional ether caused precipitation of the final product as the potassium salt, mp >300°. *Anal.* Calcd for $C_9H_7N_2O_4SK \cdot {}^1/_3\ H_2O$: C, 38.02; H, 2.72; N, 9.85. Found: C, 38.12; H, 3.18; N, 9.77. The ultraviolet spectrum at pH 12 gave an aromatic absorption with 95% of the molar extinction coefficient of 4-(2′-hydroxyphenyl)imidazole (ϵ_{max} 8420 at 252 mμ) and a phenolate anion peak of 91% (ϵ_{max} 7720 at 318 mμ). Hydrolysis at pH 4.6 gave quantitative (±3%) inorganic sulfate recovery in time intervals where less than 5% of any *O*-sulfate impurity would have hydrolyzed. The ultraviolet and the inorganic sulfate data taken together indicated that at least 97% of the compound was sulfated on nitrogen. The infrared spectrum (Nujol) showed peaks at 1230–1300 (broad multiplet), 1127 (sharp), 1111 (sharp), 1075 (sharp), 1052 (sharp), and 825 cm^{-1}.

Methanol (Baker reagent grade), D_2O (99.8% Diaprep), dioxane (purified by distillation over sodium), dimethylformamide (Fisher reagent grade), and twice-distilled deionized water were employed as solvents. Pyridine–sulfur trioxide complex (Aldrich) was stored in a desiccator at 5°. All other buffer materials were reagent grade (Baker, Fisher).

Instrumentation used in this study has previously been described (Benkovic and Benkovic, 1966). All kinetic runs were carried out in Kimax screw-cap tubes (No. 9447-A6) with Teflon-lined caps (No. 9447-B3) in a circulating water bath maintained at constant temperature (±0.1°).

The hydrolysis of 2-(4(5)-imidazolyl)phenyl sulfate in 50% v/v water–dioxane, μ = 0.2, was followed at 55, 75, and 85° by monitoring the release of 4-(2′-hydroxyphenyl)imidazole at 296 mμ. Controls indicate the product is stable during the time intervals employed, although decomposition does occur if one attempts to monitor for greater than 2 half-lives. Aliquots of the reaction mixture, 1.0–3.0 × 10^{-4} M in substrate, were withdrawn and quenched at 0° at appropriate time intervals. Duplicate runs agreed within 5%. The observed first-order rate constants for the hydrolysis were calculated from the slopes of plots of log $[(OD_\infty - OD_i)/(OD_\infty - OD_t)]$ (due to initial absorbance by substrate) against time for kinetic runs followed to at least one half-life. OD_∞ was computed from $OD_{296\ m\mu}$ of 4-(2′-hydroxyphenyl)imidazole at the corresponding pH value because of the decomposition of product after 2 half-lives. Buffers employed were 50% v/v HCl–dioxane (pH <3.4), 50% v/v formate–dioxane (pH 3.8–5.0), and 50% v/v acetate–dioxane (pH 5.2–6.1), all at μ = 0.2 (KCl). Observed rates were invariant with changing buffer concentration (formate, 0.1–0.25 M). The pH of all buffers was determined at 25°; kinetic runs with a pH variation greater than ±0.02 during the course of the run were not utilized. The observed first-order rate constants for the hydrolysis in 50% v/v deuterium oxide–dioxane, μ = 0.2, at 75° were calculated as above, the corrected pD calculated from the formula of Fife and Bruice (1961).

The hydrolytic rates of 4(5)-(2′-hydroxyphenyl)imidazole *N*-sulfate in 50% v/v water–dioxane, μ = 0.2, at 75° were determined by monitoring the release of inorganic sulfate by the barium chloranilate method of Spencer (1960). Aliquots were withdrawn and quenched at 0° at appropriate time intervals. The chloranilate absorption at 327.5 mμ was compared with a standard curve to determine the amount of inorganic sulfate present. Duplicate runs agreed within ±15%.

The pK_a values for 2-(4(5)-imidazolyl)phenyl sulfate (50% v/v water–dioxane, μ = 0.2) were determined at 25, 35, and 55° with an automatic titrator, Radiometer Model TTTlc. Estimated error at 25° and 35° is ±0.05 pK_a unit; at 55°, ±0.10 pK_a unit.

The oscillometric procedure employed was similar to that described previously (Benkovic and Benkovic, 1968). The recovery of standard inorganic sulfate solutions was quantitative (±3%); no interference from other compounds present in the solvolysis medium was noted. In mixed methanol–water solvents the amount of methyl sulfate was calculated *via* a mass balance relationship. In the presence of fluoride ions, the amount of fluorosulfate was calculated from a similar mass balance relationship. Difficulties were encountered if the ionic strength of the solution was greater than 0.5–0.6 or if the inorganic sulfate concentration was less than 0.10 mmole.

Results

The pH–rate profile for the hydrolysis of 2-(4(5)-imidazolyl)phenyl sulfate in 50% v/v dioxane–water, μ = 0.2, at 75° is shown in Figure 1. Pertinent kinetic data are summarized in Table I. Studies on salicyl sulfate (Benkovic and

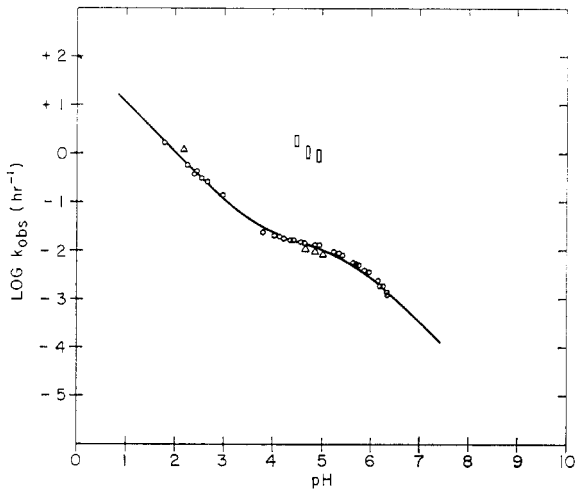


FIGURE 1: The log k_{obsd}–pH rate profile (75°, μ = 0.2) for the hydrolysis of 2-(4(5)-imidazolyl)phenyl sulfate in 50% v/v dioxane–water (○) and 50% v/v dioxane–D_2O (△). The solid line is calculated from eq 1 and data given in Table I. Points designated □ refer to the hydrolysis of 4(5)-(2′-hydroxyphenyl)imidazole *N*-sulfate (75°, μ = 0.2) in 50% v/v dioxane–water.

TABLE I: Rate Constants and Activation Parameters for the Hydrolysis of 2-(4(5)-Imidazolyl)phenyl Sulfate.

Solvent (50% v/v)	$10^2 k_{H^+}$ (M^{-1} hr^{-1})	$10^{-2}k_0$ (hr^{-1})	$\Delta H^{\pm}$ (kcal/mole^{-1})[a,b]	$\Delta S^{\pm}$ (eu)[a,b]
H_2O–dioxane	1.12[c]		25.9	+8.6
D_2O–dioxane	1.86[d]			
H_2O–dioxane		1.26[c,e]	28.0	+1.4
D_2O–dioxane		1.04[c,f]		

[a] Calculated from the equations $\Delta H^{\pm} = E_a - RT$; $\Delta S^{\pm} = (\Delta H^{\pm} - \Delta F^{\pm}/T)$; $\Delta F^{\pm} = -RT \ln k_r h/kT$ at 75°. See Figure 2 for plots of log k_0 or k_{H^+} *vs.* $1/T$. [b] Estimated error in $\Delta H^{\pm} = \pm 1$ kcal mole^{-1}; $\Delta S^{\pm} = \pm 3$ eu. [c] Calculated from eq 1. [d] Calculated from plot of log k_{obsd} *vs.* pD. [e] Kinetically determined $pK_a' = 5.40$ (75°, 50% v/v H_2O–dioxane, μ = 0.2. [f] Estimated $pK_a' = 5.91$ (75°, 50% v/v D_2O–dioxane, μ = 0.2).

Benkovic, 1968) have shown that the shape of the pH–rate profile is unaffected by the presence of dioxane, but the rate is increased some 12-fold. The pH–rate profile may be divided into two regions: (1) at pH <3, log k_{obsd} is linear with pH (slope = −1), indicative of hydronium ion catalysis; (2) at pH 4–7, log k_{obsd} is independent then dependent on pH (slope = −1) as expressed by the function $a_H/(K_a' + a_H)$, suggesting involvement of the neighboring imidazolyl group. Values of k_{obsd} may be calculated from the equation:

$$k_{obsd} = k_{H^+}a_H\left(\frac{a_H}{K_a' + a_H}\right) + k_0\left(\frac{a_H}{K_a' + a_H}\right) \quad (1)$$

which provides a satisfactory fit of the data as shown in Figure 1. A satisfactory fit of the data is also obtained by assuming the plateau rate is associated with a bimolecular term, $k_0'a_HK_a'/(K_a' + a_H)$, where $k_0' = 3.16 \times 10^3$ M^{-1} hr^{-1}. Figure 1 also gives several points showing the rate of hydrolysis of 4(5)-(2′-hydroxyphenyl)imidazole *N*-sulfate in 50% v/v dioxane–water, μ= 0.2, at 75°.

Plots of the logarithms of k_{H^+} (M^{-1} min^{-1}) and k_0 (min^{-1}) *vs.* $1/T$ are given in Figure 2 for 2-(4(5)-imidazolyl)phenyl sulfate.

The results of the solvolyses of 2-(4(5)-imidazolyl)phenyl sulfate and 4(5)-(2′-hydroxyphenyl)imidazole *N*-sulfate in mixed methanol–water at 75° are recorded in Table II. Total hydrolysis (>10 half-lives) in 50% v/v dioxane–water for *O*-sulfate at pH 2–3 and for *N*-sulfate at pH 4–5 showed theoretical (±3%) inorganic sulfate recovery. Hydrolysis of *O*-sulfate at pH 4–6 was carried out to at least 4 half-lives and was consistent with theoretical values calculated from ultraviolet spectra of the compound after a similar number of half-lives.

Figure 3 shows the results of pK_a determinations on the *O*-sulfate in 50% v/v dioxane–water, μ= 0.2, at 25, 35, and 55°. Extrapolation of the data to 75° yields a pK_a value of 5.37 ± 0.1, which is very close to the kinetically determined pK_a of 5.40. From the pK_a values a ΔH of ionization for the imidazolyl moiety was calculated to be 8.0 kcal/mole, which was found to compare favorably with those previously recorded for imidazole and imidazole compounds (Bruice and Benkovic, 1964; Nozaki *et al.*, 1957).

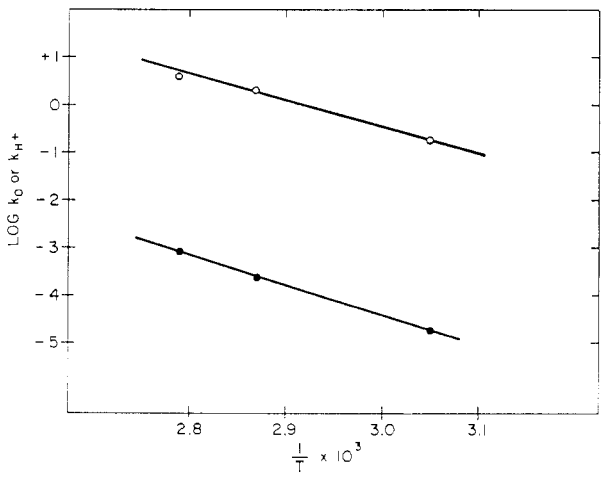


FIGURE 2: Plots of the logarithms of k_{H^+} (M^{-1} min^{-1}) (○) and k_0 (min^{-1}) (●) *vs.* $1/T$ (55, 75, 85°) for 2-(4(5)-imidazolyl)phenyl sulfate (μ = 0.2, 50% v/v dioxane–water).

Discussion

Hydronium Ion Catalysis. Hydronium ion catalysis of aromatic sulfate ester hydrolysis has long been known. Several results from the extensive studies of Burkhardt *et al.* (1936a–c) are pertinent to this discussion: (1) the rate of hydronium ion catalyzed hydrolysis of a series of 23 substituted phenyl sulfates increases with increasing electron withdrawal ($\rho \cong +0.5$); (2) the activation entropies for the series are zero or slightly positive; and (3) large substituents

TABLE II: Solvolysis of 2-(4(5)-Imidazolyl)phenyl Sulfate, 4(5)-(2′-Hydroxyphenyl)imidazole *N*-Sulfate, and Sulfur Trioxide in Aqueous Methanol.

Substrate	Mole Fraction of MeOH in Solvent	Mole Fraction of Me_2SO_4 in Product	Me_2SO_4:MeOH Ratio	Conditions, pH, μ, t (hr), Temp (°C)
2-(4(5)-Imidazolyl)phenyl sulfate	0.313	0.45	1.44	1.87, 0.32, 12, 75
	0.493	0.84	1.70	4.25, 0.30, 179, 75
4(5)-(2′-Hydroxyphenyl)imidazole *N*-sulfate	0.327	0.59	1.80	4.32, 0.35, 12, 75
	0.493	0.80	1.63	5.26, 0.25, 6, 75
Sulfur trioxide	0.303	0.57	1.88	Not given, 0.05–0.16, not given, 25

(Cl, NO_2) in the *ortho* position may act to decrease the rate one-threefold. A corollary of 1 is the relationship between the rate for hydronium ion catalyzed hydrolysis of aryl sulfates at 48.6° and the pK_a of the departing phenolic moiety according to the expression.

$$\log k_{H^+} = -1.53 - 0.25\ pK_a \qquad (2)$$

(see Figure 4). Applying the above equation to the hydronium ion catalyzed hydrolysis of 2-(4(5)-imidazolyl)phenyl sulfate—in this pH region the imidazole moiety exists as the protonated species—leads to a predicted value of log k_{H^+} = −3.72. The pK_a employed in this calculation (8.62) is based on the pK_a of *o*-$CH_2NH(CH_3)_2C_6H_4OH$ (Epstein *et al.*, 1964) in order to accurately simulate the experimentally unattainable dissociation of the phenolic hydroxyl prior to neutralization of the charged imidazolium moiety. Extrapolating the experimental value of k_{H^+} to the requisite temperature and correcting for the 12-fold accelerating effect of dioxane yields an experimental value of log k_{H^+} = −3.80. The near identity of predicted and experimental values—the deviation is less than that found for *o*-Cl substitution—suggests that no unusual electronic or steric effects are operational during hydronium ion catalyzed hydrolysis. The positive entropy term for k_{H^+} ($\Delta S^{\ddagger}$ = +8.6 eu) and the kinetic deuterium solvent isotope effect ($k_{H^+}^{H_2O}/k_{H^+}^{D_2O}$ = 0.6) support the previously postulated A-1 mechanism for hydronium ion catalyzed hydrolysis of aryl and alkyl sulfate esters (Kice and Anderson, 1966; Fendler and Fendler, 1968; Batts, 1966; Benkovic, 1966). In general the A-1 mechanism, which involves rapid preequilibrium protonation of substrate followed by rate

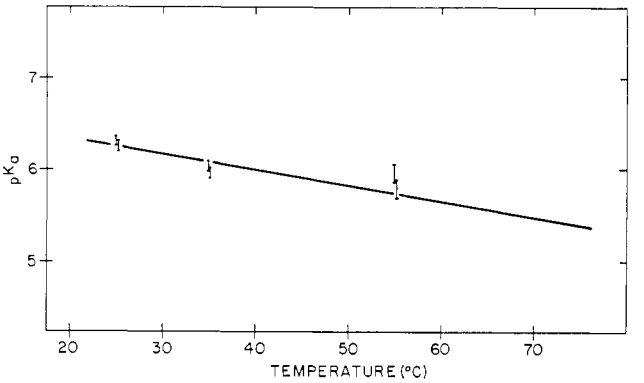


FIGURE 3: Plot of temperature *vs.* pK_a (in μ = 0.2, 50% v/v dioxane–water) for 2-(4(5)-imidazolyl)phenyl sulfate. Extrapolation to 75° gives a pK_a value which is in agreement with the experimental data of Figure 1 and Table I.

determining unimolecular decomposition, is characterized by a positive $\Delta S^{\ddagger}$ term whereas the related A-2 mechanism, which involves rate determining attack by solvent on the protonated intermediate, exhibits a $\Delta S^{\ddagger}$ ranging from −20 to −30 eu (Long *et al.*, 1957). The more negative entropy of activation presumably is a consequence of the restriction of the degrees of freedom of solvent molecules in the transition state. The greater involvement of solvent in A-2 relative to A-1 reactions is also manifested in the kinetic deuterium solvent isotope effect (k^{H_2O}/k^{D_2O}) which for reactions classified as A-2 ranges from 0.6 to 0.7 and for A-1 is <0.5 (Bunton and Shiner, 1961). The borderline behavior of k^{H_2O}/k^{D_2O} recalls similar behavior observed for salicyl sulfate and may reflect a perturbation by the *ortho* substituent of the disappearance or reorganization of hydrogen bonds in the transition state. Some support for this contention is found from the data on solvolysis of 2-(4(5)-imidazolyl)phenyl sulfate in mixed methanol–H_2O solvents (Table II) that reveal the hydronium ion catalyzed solvolysis yields a significantly

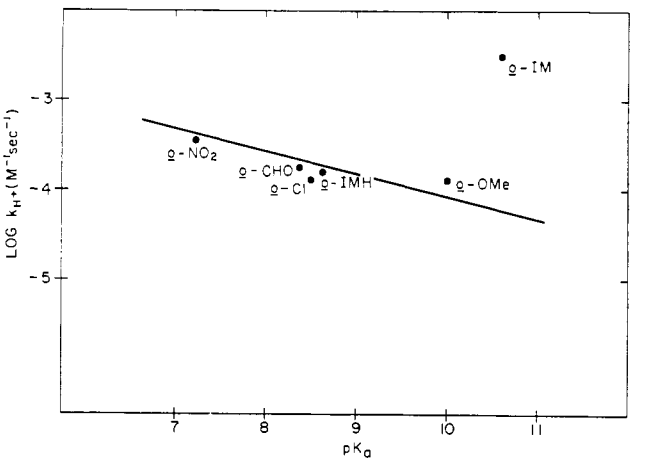
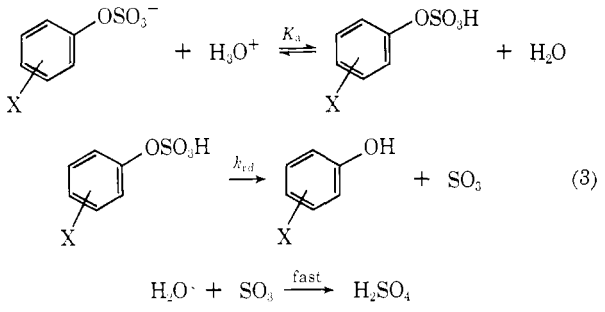


FIGURE 4: Plot of log k_{H^+} for the hydrolysis (at 48.6°) of a series of substituted phenyl sulfates *vs.* the pK_a of the corresponding phenols (Burkhardt *et al.*, 1936a–c). Solid line represents *meta* and *para* substituted substrates (see eq 2); *ortho* substituted compounds of interest are indicated individually.

lower fraction of methyl sulfate than does sulfur trioxide solvolysis (Benkovic and Benkovic, 1968). The latter is the presumed reactive initial product of the solvolysis. A reduced fraction of methyl sulfate has also been encountered in salicyl sulfate solvolysis in methanol–water mixtures in contrast to the solvolysis of *p*-carboxyphenyl sulfate which yields a product distribution identical with that obtained from sulfur trioxide (Benkovic and Benkovic, 1968). In the latter case k^{H_2O}/k^{D_2O} is also "normal." A related phenomenon is encountered in the solvolysis of a series of *ortho* and *para* substituted phenyl phosphates in alcohol–water mixtures for which the product distribution appears to depend on the presence or absence of *ortho* groups (Bunton *et al.*, 1967). The nature of this specific effect is not presently understood, but on the other hand the above data do not support an A2 type mechanism.

The accumulated evidence therefore appears to be in accord with a mechanism involving preequilibrium protonation of the ester followed by rate-determining elimination of sulfur trioxide, probably with an accompanying reorganization of solvent molecules, through the sequence of events depicted below.



It should be pointed out that the entropy of activation and solvent deuterium isotope effect alone do not distinguish between the conventional A-1 mechanism above and one involving rate-limiting proton transfer (Fullington and Cordes, 1964; Kresge and Preto, 1965; Bunton and DeWolfe, 1965). However, in the present case the rate constant for protonation of the sulfate moiety may be calculated from:

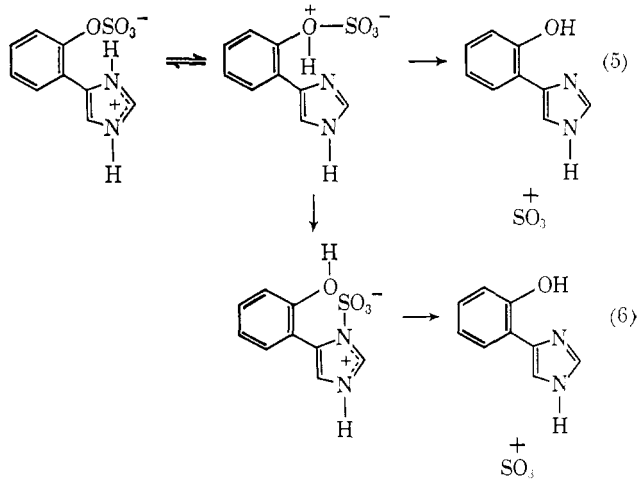
$$ROSO_3^- + H_3O^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ROSO_3H + H_2O \qquad (4)$$

where $k_1 = k_{-1}/K_a'$. Assuming k_{-1} to be diffusion controlled, 10^{11} sec^{-1} (this order of magnitude is observed for proton transfers involving strong acids in aqueous solution (Bell, 1959; Eigen, 1964), an untenable value of K_a' must be employed in order for k_1 to approach k_{H^+} ($K_a = 10^{11}/10^{-6} = 10^{+17}$). Further, the negative ρ for these reactions may only be rationalized in terms of the kinetic importance of the second step in eq 3. The classical A-1 mechanism is therefore favored, and concurs with arguments based on microscopic reversibility (Kaiser *et al.*, 1963), since there is some evidence that sulfur trioxide may be the reactive species in aromatic sulfation (Gold and Satchell, 1956; Burwell, 1952).

Intramolecular Catalysis. The second section (pH 4–7) of the pH–rate profile for 2-(4(5)-imidazolyl)phenyl sulfate may be explained in terms of intramolecular catalysis. Returning to the data of Burkhardt (Figure 4) and employing eq 2 to predict the rate of acid-catalyzed hydrolysis for the unprotonated form of 2-(4(5)-imidazolyl)phenyl sulfate, one calculates that log $k_{H^+}^{calcd} = -4.20$ (assuming a p$K_a = 10.6$ (Schmir and Bruice, 1958)) contrasted to an experimental value of log $k_0' = -2.51$, thus indicating a 49-fold rate enhancement ($k_0'/k_{H^+}^{calcd}$). Since no unusual steric effect was noted in hydrolysis *via* hydronium ion catalyzed hydrolysis, it is unlikely that any such effect is now important in this pH region. Furthermore the ratio of k_0' to k_{H^+} is 28.2 (Table I). Recall that k_0' is a second-order rate constant associated with hydronium ion catalyzed hydrolysis of the free base form of the sulfate ester. Intuitively the cationic imidazolium moiety should be electron withdrawing relative to the free base species and, hence, it is anticipated that $k_{H^+} > k_0'$ (owing to the positive ρ for the hydrolysis reaction) contrary to that observed.

Several mechanisms may be invoked to describe participation by the neighboring imidazole moiety. The kinetic solvent deuterium isotope effect $k_0^{H_2O}/k_0^{D_2O}$ of 1.2 and the positive $\Delta S^{\pm} = 1.4$ eu favor a unimolecular process rather than involvement of a mechanism featuring general base catalysis. Alternatively, the kinetic parameters may be calculated in terms of k_0' which gives $\Delta S^{\pm} = -9.8$ eu and $k_0'^{H_2O}/k_0'^{D_2O} = 0.37$ (where $k_0'^{H_2O}/k_0'^{D_2O} = (k_0^{H_2O}/k_0^{D_2O})(K_a'^{D_2O}/K_a'^{H_2O})$, with $K_a'^{D_2O}$ estimated at 1.23×10^{-4} from the pK_D of other imidazole compounds (Li *et al.*, 1961). Hence the above conclusions are not materially affected.

The possible mechanisms fall into two general categories with the imidazole group functioning either as a general acid (eq 5) or as a nucleophilic species (eq 6).



Although the imidazole moiety appears to act as a general acid–base catalyst in many enzymic reactions and their models, it also may function as a nucleophile in reactions with activated acyl groups (Jencks, 1969; Bruice and Benkovic, 1966). In order to investigate the possibility of an imidazole–sulfur trioxide intermediate, 4(5)-(2′-hydroxyphenyl)imidazole *N*-sulfate was prepared. Figure 1 illustrates that in the pH region under consideration the hydrolysis of the *N*-sulfate is some 70-fold faster. Hence, attempts to spectroscopically detect accumulation of *N*-sulfate during the hydrolysis of *O*-sulfate fail. In Table II are tabulated the results of methanol–water solvolyses. From the data it is evident that the

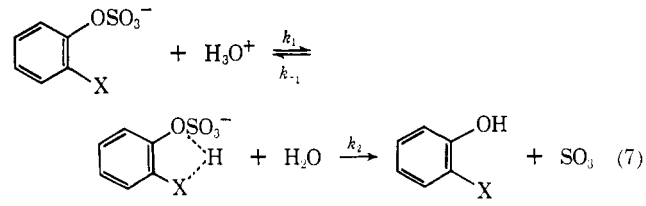
solvolyses of *O*- and *N*-sulfates yield identical product distributions, and therefore experiments designed to detect the intermediate through solvolytic behavior are inconclusive. The product ratios, however, are identical with those found in the solvolysis of sulfur trioxide in various methanol–water mixtures; hence, one can conclude that both hydrolyze *via* a transition state which generates a solvated sulfur trioxide monomer.

Further attempts to clarify the mechanism of participation involved the employment of sodium fluoride as a trapping agent. Fluoride ion reacts with *N*-sulfates, but not *O*-sulfates, to yield fluorosulfate (Fleischfresser and Lauder, 1962). Oscillometric determination of inorganic sulfate was compared to the theoretical value for complete hydrolysis and the difference was presumed to be fluorosulfate. Hydrolysis of the *N*-sulfate at 75° in the presence of 0.1 M NaF in 50% v/v dioxane–water resulted in a 23% yield of fluorosulfate. Under identical conditions hydrolysis of the *O*-sulfate releases the amount of inorganic sulfate which corresponds to the calculated percentage of hydrolysis.[1] Owing to the low efficiency of fluoride ion relative to water in trapping the *N*-sulfate, and the inherent errors in the oscillometric method, the possibility of the *N*-sulfate intermediacy cannot be decisively eliminated. However, we conclude that at least 50% of the hydrolysis reaction does not proceed through the *N*-sulfate, and for reasons discussed below, believe that the major pathway for hydrolysis does not involve this intermediate.

We now wish to compare the present study with the hydrolysis of salicyl sulfate (Benkovic, 1966). The mechanism of the carboxyl group participation reaction appears to involve intramolecular general acid catalyzed sulfur trioxide expulsion (Benkovic and Benkovic, 1968).[2]

The ratio of the rate of hydrolysis of salicyl sulfate to 2-(4(5)-imidazolyl)phenyl sulfate in terms of k_0, the unimolecular rate constant, is *ca.* 4×10^3 in 50% v/v dioxane–water and temperature insensitive. Alternatively, in terms of the bimolecular rate constant k_0', the ratio is *ca.* 47. The decrease in the magnitude of the value of the rate constants (either k_0 or k_0') with increasing pK_a suggests that the mechanism of general acid catalyzed hydrolysis (eq 5) found for salicyl sulfate extends to the present substrate. On the other hand, a mechanism involving rate-determining formation of the *N*-sulfate intermediate (eq 6) predicts an unchanged rate of hydrolysis (k_0') owing to compensation between decreased preequilibrium zwitterion formation and increased rate of formation of the *N*-sulfate intermediate.

Let us now analyze the consequence of an intramolecular general acid catalysis mechanism. Consider the following general formulation of the mechanism. It is reasonable to postulate that the proton transfer is nearly complete in the transition state (k_2 step) since (1) the deuterium solvent isotope effect measured for either k_0 or k_0' is consistent with



(7)

preequilibrium zwitterion formation and (2) a calculation similar to that for hydronium ion catalysis confirms that the rate of proton transfer to form the intermediate zwitterion is much greater than the overall rate of hydrolysis and therefore is not rate determining. An analogous situation is encountered in the hydrolysis of phosphate monoester monoanions which apparently proceeds through preequilibrium proton transfer to form the zwitterion, followed by rate-determining expulsion of monomeric metaphosphate (Kirby and Varvoglis, 1967). For these compounds, zwitterion formation becomes partially rate determining only with good leaving groups such as 2,4-dinitrophenol (Kirby and Varvoglis, 1967), further substantiating the above arguments.

In terms of the above model, the initial ground state may be described as hydronium ion plus the free base species of the *ortho* group. This reasoning implies that the additional rate terms discovered in the hydrolyses of salicyl sulfate and 2-(4(5)-imidazolyl)phenyl sulfate may arise from the greater stability of the zwitterions formed in the pH regions described, relative to those generated during hydronium ion catalyzed hydrolysis. Moreover, differences in rate between the two substrates may also be ascribed to a difference in zwitterion concentration.

A major source of possible zwitterion stabilization is hydrogen bonding. Existence of a hydrogen bond in salicylic acid anion in water is implied by differences between the pK_a values for the phenolic dissociation of the *ortho* and *para* isomers (o = 13.60, p = 9.38) (Kortum *et al.*, 1961). A similar but weaker hydrogen bond is probably present in the free base form of 4(5)-(2′-hydroxyphenyl)imidazole, on the basis of a smaller change in the pK_a values, *ortho* = 10.6 (Schmir and Bruice, 1958), *para* estimated at 9.4 (assuming the imidazole moiety to exhibit an electronic effect similar to carboxylate anion). Since thermodynamic measurements possess an inherent ambiguity, a more direct measure of the presence of such intramolecular hydrogen bonds is indicated by a comparison of the recombination constants for the system:

$$HO^- + H^+ \cdots \text{substrate} \xrightarrow{k_R} H_2O + \text{substrate}$$

(Eigen and Kruse, 1963). For a phenolic substrate with an *ortho* nitro group, $k_R = 1.1 \times 10^{10}$ M^{-1} sec^{-1}, approaching diffusion control, whereas with *ortho* carboxylate groups $k_R = 1.1 - 5.3 \times 10^7$ M^{-1} sec^{-1}, manifesting a neighboring group interaction. It is reasonable to assume that differences in pK_a (ΔpK_a) between particular *ortho* and *para* substituted phenols arise mainly from variations in k_R owing to hydrogen bonding, and hence that such differences might be directly manifested in lowering k_{-1} of the preequilibrium step in eq 7. The greater the ΔpK_a, the more efficient the catalysis expected by the *ortho* substituent, owing to an increase in the concentration of the zwitterionic species.

[1] Control experiments under identical conditions of pH, temperature, and solvent demonstrated that sodium fluorosulfate was stable during time intervals employed.

[2] In unpublished experiments the hydrolysis of salicyl sulfate was conducted in the presence of hydroxylamine at concentrations where an acyl sulfate intermediate would be efficiently converted to the salicyl hydroxamic acid. No evidence for such a species was found. (Conditions: 10^{-2} M salicyl sulfate, 0.8 M hydroxylamine HCl, pH 3.12–3.49.)

TABLE III: $\Delta(\Delta F^{\ddagger})$ for Sulfate and Phosphate Ester Hydrolysis *vs.* $\Delta(pK_a)$ of the Corresponding Phenols.

Substrates	$\Delta(\Delta F^{\ddagger})^{a,b}$	Phenolic pK_a[c]	ΔpK_a
2-(4(5)-Imidazolyl)phenyl sulfate	1.90	10.6 ± 0.2	1.2 ± 0.4
4-(4(5)-Imidazolyl)phenyl sulfate		9.4 ± 0.2	
2-(4(5)-Imidazolyl)phenyl sulfate	1.88	10.60 ± 0.20	1.98 ± 0.25
2-(4(5)-Imidazolyl)phenyl sulfate, protonated		8.62 ± 0.05	
2-(4(5)-Imidazolyl)phenyl sulfate	2.44	10.60 ± 0.20	3.00 ± 0.40
Salicyl sulfate		13.60 ± 0.20	
Salicyl phosphate	3.55	13.60 ± 0.20	4.22 ± 0.27
4-Carboxyphenyl phosphate		9.38 ± 0.07	
Salicyl sulfate	3.74	13.60 ± 0.20	4.22 ± 0.27
4-Carboxyphenyl sulfate		9.38 ± 0.07	
2-(4(5)-Imidazolyl)phenyl sulfate, protonated	4.32	8.62 ± 0.05	4.98 ± 0.25
Salicyl sulfate		13.60 ± 0.20	

[a] A 5% error in each rate constant would give an error of ±0.05 in $\Delta(\Delta F^{\ddagger})$. [b] All calculated from second-order rate constants except for the phosphates, which are unimolecular. $\Delta(\Delta F)$ is insensitive to minor variations in electronic effects. [c] All at 25°, from Kortum *et al.*, 1961 and Albert and Serjeant, 1962.

The data in Table III and Figure 5 indicate a linear relationship between $\Delta(\Delta F^{\ddagger})$ and ΔpK_a which supports the above arguments. The bottom line in Figure 5 is a replotting of the Burkhardt data of Figure 4 for the hydronium ion catalyzed hydrolysis of aryl sulfates which exhibit no additional intramolecular term (Burkhardt *et al.*, 1936a–c). The upper line is obtained from compounds with substituents capable of hydrogen bonding on the basis of ΔpK_a and k_R criteria; comparisons are made only between phenols with substituents whose electronic effects are considered to be similar. The reasonable fit of the data suggest that the differences observed between not only intramolecular general acid catalysis and hydronium ion catalysis but also between two intramolecular reactions may be rationalized in terms of hydrogen bonding leading to zwitterionic stabilization. It appears necessary, however, that both k_R and ΔpK_a criteria be applied, since the hydrolysis of *o*-CHO phenyl sulfate is not accelerated relative to the Burkhardt series, although a ΔpK_a can be detected upon comparison of the isomeric *ortho* and *para* phenols. In this case k_R is $>10^{10}$, suggesting that ΔpK_a here is sensitive to the conformation assumed by the aldehyde moiety. Thus, it should be stressed that the empirical correlation probably is only valid if ΔpK_a is a true reflection of k_R for the *ortho* substrate.

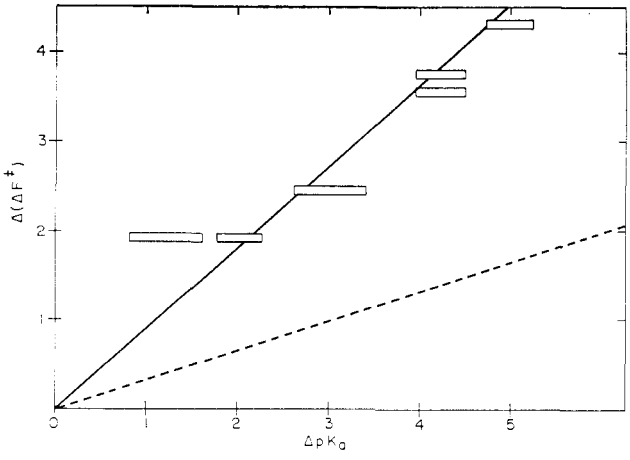


FIGURE 5: Plot of $\Delta(\Delta F^{\ddagger})$ for sulfate and phosphate ester hydrolysis *vs.* $\Delta(pK_a)$ of the corresponding phenols. Points refer to substrates listed in Table III.

Although we have viewed the above mechanism as involving a discrete zwitterion intermediate, it should be stressed that the intermediate is metastable and resembles, except for the development of S–O bond scission, the transition state for hydrolysis. The possible stabilization of the transition state owing to hydrogen bonding should be considerably greater than similar stabilization of ground-state complexes, presumably because the developing basicity or acidity of a transition state can exceed that which can exist in ground states at equilibrium (Jencks, 1969). This phenomenon may be attributed to insufficient solvation of the transition state complex. For the two systems discussed, the intramolecular hydrolysis might therefore be alternatively described in terms of a concerted process. The empirical relationship can thus be explained in terms of two models which differ only in the position of the proton. Although the position of the proton may be only of academic interest, it cannot be ascertained with certainty by any of the experimental criteria.

The ΔpK_a rationale simply reduces then to a relationship between product structure and transition state stability as manifested by the former's lower free energy owing to hydrogen bonding. The rate acceleration observed for the hydrolysis of 2-(4(5)-imidazolyl)phenyl sulfate indicates that an imidazole moiety fulfills the necessary chemical requirements to be present at the active site of arylsulfatase. On purely chemical grounds, the possible rate acceleration afforded by the neighboring carboxyl group is much greater ($k_0'/k_{H^+}^{calcd}$ = 1.1×10^4 for salicyl sulfate; $k_0'/k_{H^+}^{calcd}$ = 49.0 for 2-(4(5)-imidazolyl)phenyl sulfate). It is noteworthy but certainly not conclusive that the enzymic activity of arylsulfatases is maximal at pH 4–5.

The two present models, salicyl sulfate and 2-(4(5)-imida-

zolyl)phenyl sulfate, hydrolyze *via* six-membered transition states. Owing to the unique chemistry of phosphate esters in potential five-membered ring systems, our future investigations on sulfates will center on such models.

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